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Impedance Biosensor Utilizing a Si Substrate Deposited by Wet Methods

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Abstract

We report an impedance biosensor utilizing a Si electrode created by wet chemical deposition atop 6061 Al alloy. The sensor electrode is created by galvanic/electroless Si deposition from an electrolyte containing 10 mM HF and 20 mM Na_2SiF_6 in 80 wt% formic acid, followed by antibody immobilization. The impedance response of the sensor electrode to increasing concentrations of peanut protein Ara h 1, a common food allergen, can be fit to an equivalent circuit containing three RC loops. The circuit element most sensitive to antigen binding is the charge transfer resistance, yielding a detection limit of 4 ng/mL.

Biosensors that utilize electrochemical impedance spectroscopy have been employed with a wide variety of immobilized biomolecules, including antibodies, receptor proteins, aptamers, and ssDNA.[1-3] These biomolecules must be immobilized atop a conductive and biocompatible substrate, which is most commonly accomplished by amide bond formation to carboxylate-terminated Au-thiol self-assembled monolayers.[4] However, Au-thiol self-assembly chemistry has been reported to have inadequate stability for many applications, with a shelf life limited to days to weeks.[5] In addition, most sensors need to be calibrated, which for antibody-based biosensors requires antibody unfolding. For this reason, durable chemistry for antibody immobilization is also needed for biosensor regeneration during such a calibration procedure.

In addition to Au, other biocompatible substrate materials that have been employed for impedance biosensors include C,[6,7] Si,[8-10] Pt,[11,12] Ti,[13,14] and ITO.[15,16] Si is intriguing as a biosensor substrate, since it is directly below C in the periodic table, so Si-C bonds are of comparable strength to C-C and Si-Si bonds. Additional advantages of Si substrates for biosensors include easier incorporation into ULSI devices and easier surface preparation relative to C. Room temperature combined galvanic and electroless deposition of compact Si films was recently reported from concentrated formic acid.[17] Here these Si films are used for immobilization of the mouse monoclonal antibody to peanut protein Ara h 1, and subsequent impedance detection of the protein antigen. Peanuts are considered one of the most dangerous food allergens, with severe anaphylactic reactions causing over 100 fatalities in the United States alone each year.[18] Nine possible allergens within peanuts have been identified, Ara h 1 to Ara h 8, and peanut oleosin,[19,20] with Ara h 1 the most widely studied.

Experimental

Semiconductor grade 10 wt% HF was obtained from J.T. Baker; Al 6061 alloy was obtained from McMaster Carr; Na_2SiF_6, formic acid, and 10-undecenoic acid were obtained from Alfa Aesar; N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and toluene were obtained from Acros Organics; phosphate buffer saline (PBS) was obtained from A.A Hoefer; potassium hexa-cyanoferrate(III) was obtained from Sigma Aldrich; N-hydroxysulfosuccinimide sodium salt (NHSS) was obtained from Thermo Scientific; and peanut protein Ara h1 and its monoclonal antibody were obtained from Indoor Biotechnologies. All reagents were used as received. The Ara h1 protein was labeled as the monomeric form.

Prior to Si deposition, Al 6061 electrodes were polished sequentially with 400, 600, and 1200 grit Al_2O_3 sand papers, then rinsed with acetone and distilled water according to ASTM standard B253–11. Si films were then grown atop Al 6061 alloy by 30 hr. of galvanic/electroless deposition from 10 mM HF and 20 mM Na_2SiF_6 in 80 wt% formic acid.[17] Instead of using an Al rotating disc electrode (RDE) as in prior experiments,[17] the electrolyte was agitated at 700 rpm with a magnetic stir bar. This yields Si films ~ 19 μm thick, as determined by cross-sectional scanning electron microscopy (SEM). Before and after Si deposition, the surface roughness was measured with a Mahr-Perthen Perthometer PRK stylus surface profilometer.

Following Si deposition, the substrate was immersed immediately in 10% 10-undecenoic acid in deaerated toluene solution for 17 hr.[21] The exposed carboxylic acid groups were then activated by immersion for 1 hr into 75 mM EDC, 15 mM NHSS, and 50 mM phosphate buffer

solution (PBS) at pH 7.3. Following carboxylate group activation, the antibody to peanut protein Ara h 1 was immobilized through amide bond formation by immersing the electrode for 1 hr into a solution containing 50 μg/mL antibody and 50 mM phosphate buffer solution (PBS) at pH 7.3.[22]

The Si-coated electrode was used as the working electrode in a virgin Teflon three-electrode cell with a Pt spiral counter electrode and an Ag/AgCl (1.0 M KCl) reference electrode. Impedance measurements were performed with a Gamry Instruments Reference 600 over the frequency range from 0.01 Hz to 15 kHz with an AC probe amplitude of 5 mV. Each impedance spectrum takes about 2.8 min. to acquire. To minimize the possibility of Si oxidation, the impedance results were obtained at a DC potential of 0 mV vs. Ag/AgCl, which is slightly cathodic to the open circuit potential (approximately +50 mV) in the electrolyte of interest.

Results and Discussion

As discussed above, dark grey Si films are grown atop Al 6061 alloy by 30 hr. of galvanic/electroless deposition from 10 mM HF and 20 mM Na_2SiF_6 in 80 wt% formic acid.[17] Following emersion into laboratory air, these Si deposits retain their dark grey color and appearance indefinitely, suggesting formation of compact Si films. Gradual color change from dark grey to white is typically used to indicate formation of porous Si that gradually oxidizes to SiO_2.[23] This same Si deposition chemistry was recently employed for Si nanowire formation, and amorphous Si deposition was verified by observation of the characteristic broad Raman band from 480-540 cm^{-1}.[24]

Figure 1 illustrates the Nyquist plots of the impedance response during different stages of electrode fabrication, and Figure 2 illustrates the impedance response of the sensor electrode to increasing concentrations of peanut protein Ara h 1. In Figure 2, the impedance magnitude clearly increases with the Ara h 1 concentration. Several Ara h 1 concentrations that were tested are omitted from the results in Figure 2 to make this easier to read. The results in Figures 1 and 2 are fit to the equivalent circuit of Figure 3 by complex non-linear least squares (CNLS) regression. Figure 3 contains three RC loops in series with the capacitances replaced by constant phase elements (CPE), where R_1 and CPE_1 corresponds physically to the solid-liquid interface, R_2 and CPE_2 to the polymer-protein film, and R_3 and CPE_3 to the Si film. For the bare Si film, R_2 and CPE_2 is omitted from the data fit, since the electrode is not coated with a polymer-protein film. The best-fit equivalent circuit elements are given in Tables 1 and 2.

Since the thickness, composition, and electrostatic charge characteristics of the polymer-protein film and therefore solid-electrolyte interface vary with surface preparation, the values obtained for most of these equivalent circuit elements in Tables 1 and 2 cannot be directly compared. However, the Si film is present for all of these impedance measurements, and R_3 (2-3 $k\Omega\text{-}cm^2$) does not vary substantially during the different measurements. From inspection of Table 2, the equivalent circuit element most sensitive to binding of peanut protein Ara h 1 is R_1, which is equivalent to the charge transfer resistance to Faradaic electron transfer in this system. Figure 4 plots the variation in R_1 with antigen concentration and has the shape typifying a Langmuir adsorption isotherm,[10,25] with an approximately linear response at low antigen concentrations, and a gradual approach to antibody film saturation at high concentrations.

The detection limit is determined from the following relationship:

$$Detection\,Limit = \frac{3\sigma}{Sensitivity} \quad (1)$$

where σ is the standard deviation of the charge transfer resistance (R_1) in the blank electrolyte, and the sensitivity is the slope of the linear portion of Figure 4. This yields a detection limit for Ara h 1 of 4 ng/mL. Unfortunately, this number cannot be directly compared to an exact allergenic threshold for peanut protein ingestion due to variations in response between allergic individuals.[26] However, a general guideline for the detection of food allergens has been reported as 1–100 mg/kg of food.[27] Assuming food has the same density as water, this yields an allergenic threshold of 1–100 μg/mL. The detection limit reported here is well below this range.

The values for the exponents (n_1, n_2, n_3) of the constant phase elements (CPE) in Tables 1 and 2 range from 0.50 to 0.88, and many of these values are not close to unity, as typically required when a CPE is substituted for a capacitive element. For this reason, standard tests were performed to assess the validity of the impedance spectra shown in Figures 1 and 2.[28] Impedance measurements at AC probe amplitudes ranging from 0.5-10.0 mV yield identical impedance spectra, demonstrating system linearity. Repeat measurements of the same spectrum yield identical results, demonstrating system stability. The impedance data presented in Figures 1 and 2 are also found to satisfy the Kramers-Kronig relations, the most rigorous test for the validity of impedance spectra.[28]

The low values for n_1, n_2, n_3 in Tables 1 and 2 can be explained by the high surface roughness in this system, which is measured as 0.7 and 4.8 μm, before and after Si deposition. ASTM standard B253–11 was followed for surface preparation, and this standard is designed for electrodeposition onto Al and Al alloys, where the first step is galvanic Zn deposition using zincate methods. During galvanic deposition processes in general, high surface roughness is often required in order to obtain adequate coating adhesion. During the current studies, Si deposition onto smoother Al substrates resulted in film delamination during biosensor fabrication and testing.

Conclusions

We recently reported wet chemical deposition of thick Si film by combined galvanic and electroless deposition onto Al 6061 alloy from electrolytes containing 10 mM HF and 20 mM Na_2SiF_6 in 80 wt% formic acid (HCO_2H). Here we employ these wet chemically deposited Si films as substrates for impedance biosensing of peanut protein Ara h 1. The impedance response to increasing peanut protein concentrations can be fit to an equivalent circuit with three RC loops in series, but with the capacitance values replaced with a constant phase element (CPE). The circuit element most sensitive to antigen binding is the charge transfer resistance at the electrode-electrolyte interface (R_1). The detection limit calculated from determination of R_1 is 4 ng/mL.

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Institute of Technology-Madras for helpful discussions about electrochemical impedance spectroscopy of high surface roughness electrodes, and for use of his online KKT program at http://www.che.iitm.ac.in/~srinivar/.

Table 1. Best-fit equivalent circuit parameters (standard errors) during interface fabrication.

Equivalent Circuit Element	Si	10-undecanoic acid	NHSS
R_s (Ω-cm^2)	83.7 (5.1)	28.1 (1.5)	42.1 (4.8)
T_1 (μF cm^{-2} s^{n-1})	30.88 (4.16)	16.47 (1.17)	14.3 (0.4)
n_1	0.50 (0.04)	0.50 (0.01)	0.77 (0.01)
R_1 (kΩ-cm^2)	0.96 (0.20)	6.51 (2.30)	9.80 (0.41)
T_2 (μF cm^{-2} s^{n-1})		0.44(0.22)	3.74 (0.51)
n_2		0.78 (0.08)	0.64 (0.05)
R_2 (kΩ-cm^2)		0.55 (0.22)	4.53 (0.69)
T_3 (μF cm^{-2} s^{n-1})	18.67 (1.46)	15.9 (2.2)	0.27 (0.04)
n_3	0.82 (0.04)	0.87 (0.07)	0.83 (0.03)
R_3 (kΩ-cm^2)	1.98 (0.22)	3.22 (0.97)	1.66 (0.36)

Table 2. Best-fit equivalent circuit parameters (standard errors) upon increasing exposure of antibody film to peanut antigen.

Ara h1 concentration (μg/mL)	0	0.005	0.01	0.015	0.02	0.04	0.08	0.16	0.32	0.64
R_s (Ω-cm^2)	52.9 (3.9)	52.4 (3.2)	48.5 (3.1)	46.6 (3.2)	42.4 (43.7)	41.3 (3.0)	39.0 (3.3)	34.7 (3.3)	32.0 (3.6)	32.6 (3.6)
T_1 (μF cm^{-2} s^{n-1})	12.7 (0.4)	12.4 (0.4)	12.0 (0.4)	11.6 (0.4)	11.2 (0.4)	11.2 (0.4)	10.9 (0.5)	11.1 (0.5)	11.0 (0.5)	11.2 (0.5)
n_1	0.68 (0.02)	0.66 (0.02)	0.65 (0.02)	0.65 (0.02)	0.63 (0.02)	0.64 (0.02)	0.63 (0.02)	0.63 (0.02)	0.61 (0.02)	0.62 (0.02)
R_1 (kΩ-cm^2)	14.6 (0.7)	16.0 (0.7)	16.9 (0.8)	17.1 (0.8)	18.3 (1.0)	18.3 (0.9)	18.6 (1.0)	18.9 (1.1)	19.5 (1.2)	19.6 (1.2)
T_2 (μF cm^{-2} s^{n-1})	2.62 (0.41)	2.61 (0.35)	2.54 (0.34)	2.52 (0.33)	2.55 (0.37)	2.51 (0.31)	2.66 (0.37)	2.77 (0.35)	2.76 (0.38)	2.72 (0.37)
n_2	0.72 (0.05)	0.73 (0.04)	0.73 (0.04)	0.73 (0.04)	0.74 (0.04)	0.72 (0.04)	0.72 (0.04)	0.71 (0.04)	0.72 (0.04)	0.71 (0.04)
R_2 (kΩ-cm^2)	4.42 (0.76)	4.37 (0.69)	4.47 (0.74)	4.52 (0.75)	4.38 (0.86)	4.92 (0.81)	4.82 (0.93)	5.12 (0.99)	4.97 (1.04)	5.08 (1.05)
T_3 (μF cm^{-2} s^{n-1})	0.28 (0.02)	0.29 (0.02)	0.27 (0.01)	0.28 (0.02)	0.28 (0.02)	0.27 (0.01)	0.26 (0.02)	0.27 (0.02)	0.27 (0.02)	0.26 (0.02)
n_3	0.81 (0.01)	0.81 (0.01)	0.81 (0.01)	0.81 (0.01)	0.81 (0.01)	0.81 (0.01)	0.81 (0.01)	0.81 (0.01)	0.81 (0.01)	0.81 (0.01)
R_3 (kΩ-cm^2)	2.39 (0.25)	2.37 (0.21)	2.39 (0.20)	2.37 (0.19)	2.39 (0.20)	2.41 (0.19)	2.32 (0.21)	2.31 (0.19)	2.38 (0.20)	2.30 (0.20)

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Figure captions

Figure 1. Nyquist plots of the impedance response (a) of galvanic Si deposit (■), (b) after modification of the Si surface with 10-undecenoic acid (▲), and following treatment with EDC and NHSS (●). The test solution also contains 50 mM PBS buffer and 5 mM K_3FeCN_6 + 5 mM K_4FeCN_6 at pH 7.3.

Figure 2. Nyquist plots for the impedance response of the antibody-coated electrode after exposure to 0 (◆), 0.005 (■), 0.01 (▲), 0.02 (×), 0.08 (●), 0.32 (+), and 0.64 (✳) µg/mL of peanut protein Ara h 1. The test solution also contains 50 mM PBS buffer and 5 mM $K_3Fe(CN)_6$ + 5 mM $K_4Fe(CN)_6$ at pH 7.3.

Figure 3. Equivalent circuit employed to fit the impedance results during interface fabrication, and following exposure to increasing concentrations of peanut protein Ara h 1.

Figure 4. Variation in R_1 with concentration of peanut protein Ara h 1.

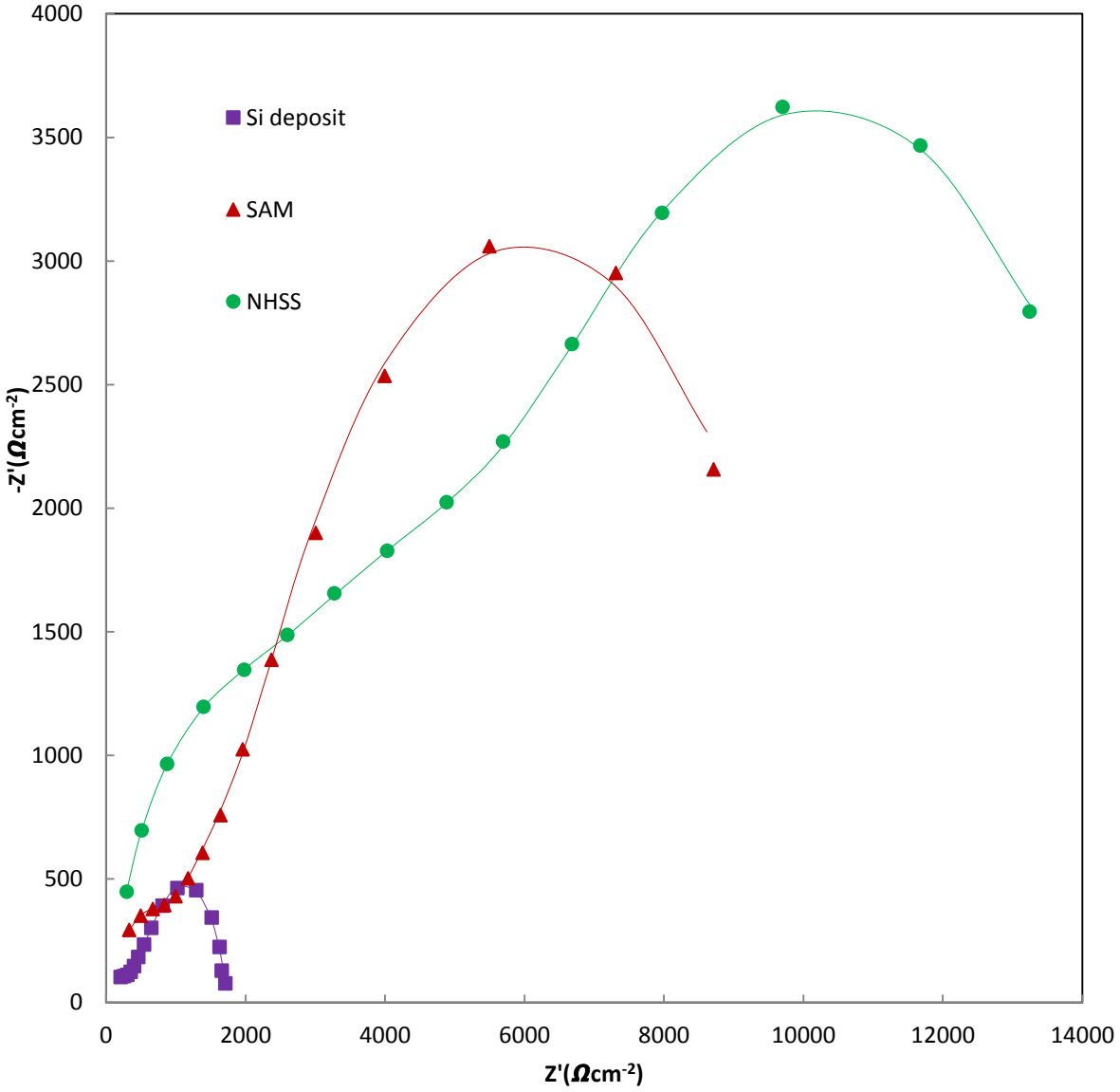
Si deposit
SAM
NHSS
-Z'(Ωcm-2)
Z'(Ωcm-2)
0
500
1000
1500
2000
2500
3000
3500
4000
0
2000
4000
6000
8000
10000
12000
14000

Figure 1.

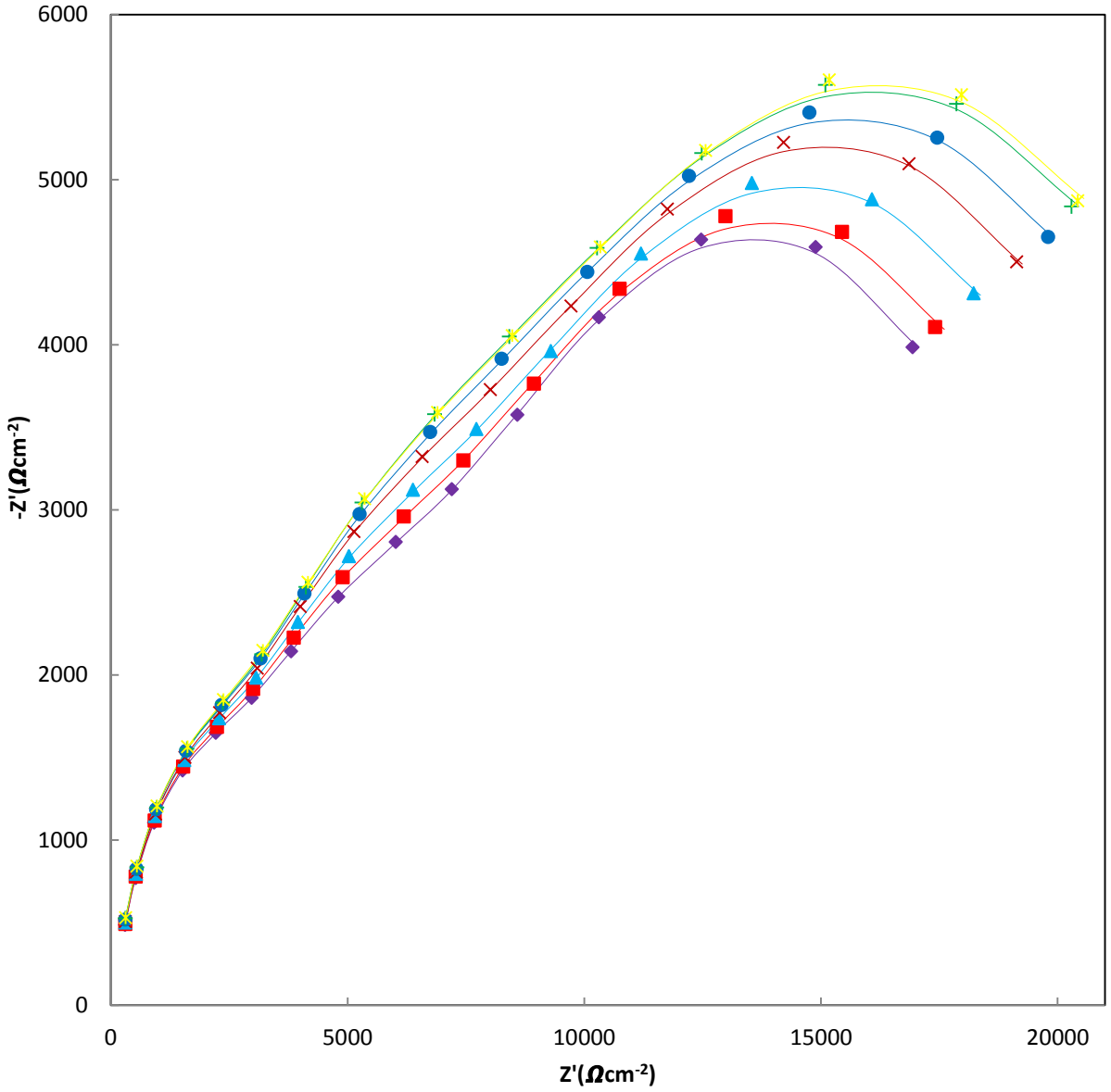
6000
5000
4000
3000
2000
1000
0
-Z'(Ωcm-2)
0
5000
10000
15000
20000
Z'(Ωcm-2)

Figure 2.

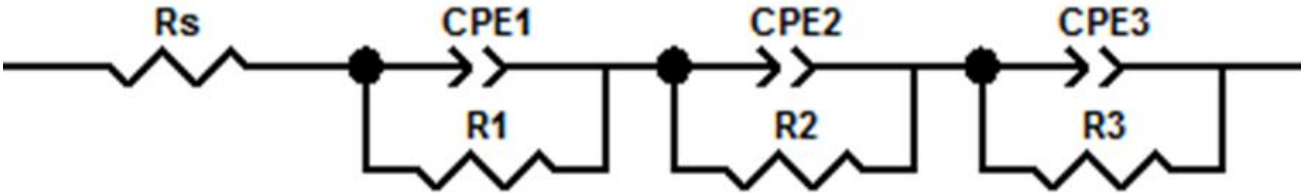

Figure 3.

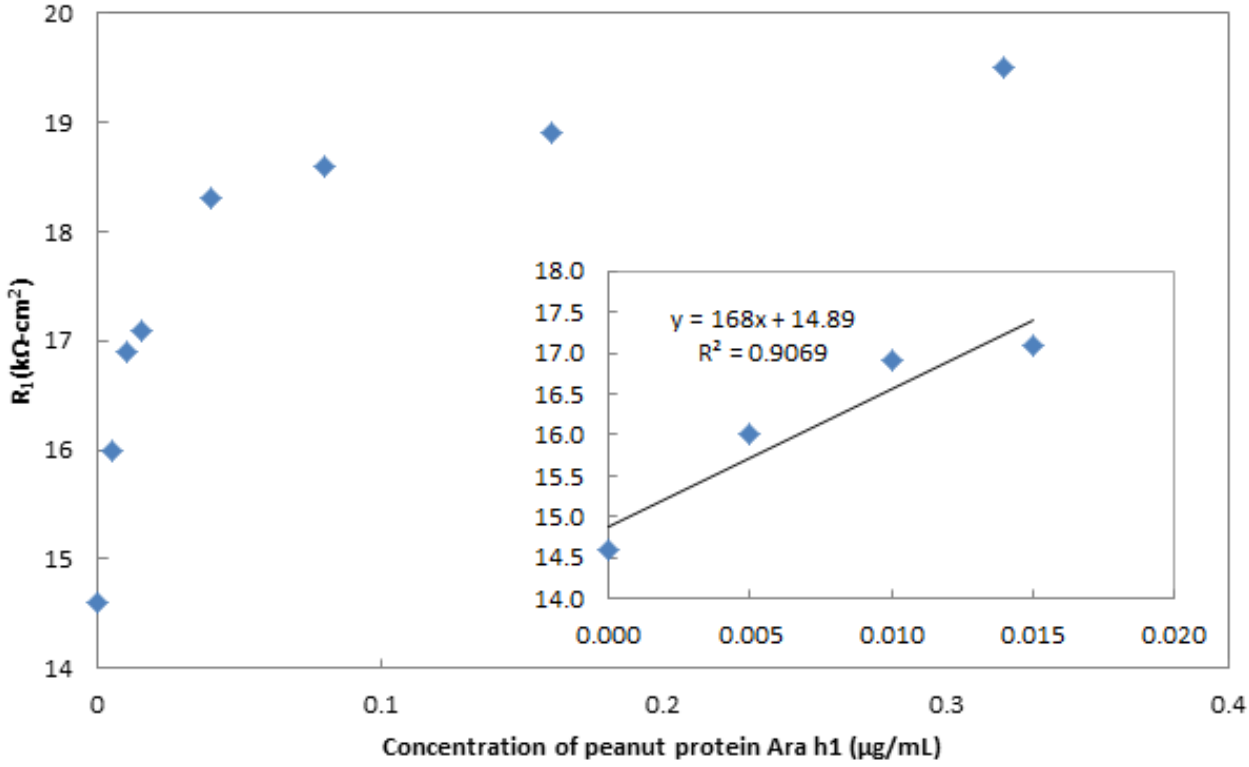
20
19
18
17
16
15
14
R_1(kΩ·cm²)
0
0.1
0.2
0.3
0.4
Concentration of peanut protein Ara h1 (μg/mL)
18.0
17.5
17.0
16.5
16.0
15.5
15.0
14.5
14.0
y = 168x + 14.89
R² = 0.9069
0.000
0.005
0.010
0.015
0.020

Figure 4.